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Phenotypic and genotypic characteristics of *Listeria monocytogenes* strains isolated during 2011-2014 from different food matrices in Switzerland

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19 **ABSTRACT**

20 One hundred and forty two *L. monocytogenes* strains isolated from different food matrices in
21 Switzerland between 2011 and 2014 were characterized with respect to their genotypic and
22 phenotypic properties. Analyzed strains originated from various meat, milk, plant-associated
23 food products and production environments as well as from other types of foods including
24 fish, seafood, and ready to eat (RTE) products. The collection included serotype 1/2a (64%),
25 4b (15%), 1/2c (12%), 1/2b (7%) and 3c (3%), as well as both genetic lineage I and II strains.
26 The strains were genetically diverse representing 61 MLST sequence types (ST) including 24
27 new ST. The most frequent clonal complexes (CC) were CC9 and CC121. PCR screening
28 detected presence of the stress survival islet (SSI-1) in 50 % of the strains. Phenotypic
29 resistance to benzalkonium chloride (BC) was detected in 18% of the strains. The BC
30 resistance genetic determinants *qacH* and *bcrABC* were detected in 80% and 12% of the
31 strains, respectively. Most (n=129) of the strains isolated from Swiss food matrices exhibited
32 poor biofilm formation capacity and there were no correlations detected between strain
33 serotypes, genotypes and biofilm production.

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1. Introduction

Listeria monocytogenes is an important foodborne pathogen with a significant impact on public health and economy worldwide. Although human infections with *L. monocytogenes* occur rarely they lead to serious and life-threatening disease conditions (listeriosis) in those with diminished immunity including septicemia, meningitis, meningoencephalitis and abortion (Dogany, 2003). Because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to foodborne illnesses (Anonymous, 2009). In the European Union there were 1,476 confirmed cases of listeriosis reported in 2011 resulting in an overall notification rate of 0.32 cases per 100,000 people (Anonymous, 2013a). In Switzerland, the annual incidence over the past ten years ranged from 0.47 to 1.22 cases per 100,000 people. So far, three Swiss listeriosis outbreaks have occurred including the latest one reported in 2011 caused by the consumption of contaminated cooked ham (Bula et al., 1995; Bille et al., 2006; Haechler et al., 2013). Althaus et al. (2013) recently characterized *L. monocytogenes* strains from human listeriosis cases that occurred in Switzerland during the period 2011-2013.

To gain insight into the relationships and further characteristics of strains occurring in foods in Switzerland, the aim of the present study was to further characterize *L. monocytogenes* strains collected by the Swiss National Reference Laboratory between 2011 and 2014 by phenotyping and genotyping methods and compare these results with data from other countries in Europe and the United States and human strains isolated in Switzerland.

2. Material and Methods

2.1 Strain selection

One hundred and forty two *L. monocytogenes* strains collected between 2011 and 2014 by the Swiss National Reference Centre for Enteropathogenic Bacteria and Listeria were characterized. This strain collection comprises isolates that were collected through elective *L. monocytogenes* screenings performed by food processing companies as well as those collected in the course of periodical inspections undertaken by the authorities.

2.2 Phenotypic characterization

Strains serotypes were determined using the commercial set of Listeria O-factor and H-factor antisera from Denka Seiken (Pharma Consulting, Burgdorf, Switzerland) according to the manufacturer's instructions. BC resistance was determined as previously described (Mullapudi et al., 2008). Briefly, overnight cultures from each strain grown in Mueller Hinton (MH) broth were spotted and incubated 48 hrs at 37°C on MH agar plates (Oxoid, Pratteln, Switzerland) with 2 % defibrinated sheep blood (Oxoid, Pratteln, Switzerland) and supplemented with different BC concentrations (0, 5, 10, 15, 20, 25, 30, 35, and 40 µg/ml). Biofilm formation capacity of the strains was assessed in Tryptone Soy Broth (TSB; Oxoid, Pratteln, Switzerland) media using the crystal violet staining method in microtitre plates as previously described by Harvey et al. (2007). Briefly, overnight cultures prepared from each strain were inoculated in triplicate on 96 well microtitre plates that included 9 control wells containing 100 µl of un-inoculated TSB (Thermo Fisher Scientific, Roskilde, Denmark) and incubated at 20 °C for 48 h. The formed biofilms were subsequently stained using 1% crystal violet solution and quantified by measuring optical density at 595 nm.

2.3 Genotypic characterization

Multi locus sequence typing (MLST) was performed as described by Ragon et al. (2008) and the alleles and sequence types (STs) determined are publicly available at www.pasteur.fr/mlst.

PCRs to determine the presence of the five genes comprising the stress survival islet (*lmo0444-lmo0448*) were performed using the primers described by Ryan et al. (2010) and the Phusion High Fidelity Taq Polymerase system (Thermoscientific, St. Leon-Rot, Germany). PCRs to determine *qacH* and *bcrABC* presence were conducted using primers described by Müller et al. (2013) and Elhanafi et al. (2010), respectively, and the Go Taq Green Master Mix (Promega, Madison, USA).

3. Results

One hundred and forty two *L. monocytogenes* strains isolated between 2011 and 2014 from foods and food-associated environments in Switzerland were characterized. An overview showing the distribution of these strains based on food source and serotypes is presented in Table 1. Of these strains, 58 % (89/142) were meat associated isolated from meat products and their production environments, 20% (34/142) were milk associated isolated from milk products and their production environments, 5% (9/142) were isolated from plant associated foods including salad, corn and rice products and 6% (10/142) were isolated from other food products including fish, seafood, quorn and RTE. Overall strains from serotypes 1/2a (n=91) and 4b (n=21) were the most frequently isolated, whereas serotypes 1/2b, 1/2c and 3c were found at lower frequency. Among the meat-associated isolates serotypes 1/2a (n=52), 1/2b (n=6), 1/2c (n=15), 3c (n=3) and 4b (n=13) were represented. Serotypes 1/2a (n=24), 1/2b (n=3), 1/2c (n=1) and 4b (n=6) were found among strains recovered from milk-associated products. Strains from plant associated food products comprised serotypes 1/2a (n=6), 1/2c (n=1) and 4b (n=2). Serotypes 1/2a (n=9) and 1/2b (n=1) were the only serotypes found among strains derived from other food products.

110 Using MLST there were 61 different sequence types (ST) detected among the 142 *L.*
111 *monocytogenes* strains analyzed including 24 newly assigned STs (ST 724 – 728, 733 and 738
112 – 755). These 61 STs were grouped into 24 clonal complexes (CC) and 6 singletons (Table 2).
113 ST9 (n=18) and ST121 (n=14) formed the largest groups. ST2 and ST204 comprised seven
114 strains, ST155 six strains, ST6 and ST8 five strains, ST3 and ST504 four strains and ST16,
115 ST230, ST415 and ST415 three strains. The rest of the STs were all represented by either two
116 or one strains. ST9 included serotype 1/2c (n=13), 1/2a (n=3) and 3c (n=2) strains; ST121
117 1/2a (n=13) and 3c (n=1) strains and ST155 serotype 1/2a (n=5) and 1/2c (n=1) strains. Other
118 STs that also contained more than one strain all comprised of a single serotype. Apart from
119 serotype 1/2c, which was isolated more often from meat associated products than others there
120 was no significant correlation found between genetic lineage, serotype or clonal complex and
121 the matrix origin of the strains.

122 The SSI-1 was detected in 50% (71/142) of the strains representing a variety of MLST
123 sequence types (Table S1). In general all strains within a given CC either harbored or lacked
124 SSI-1. An exception to this observation was only detected within CC9 where one strain was
125 negative SSI-1. In 5 serotype 1/2a strains assigned to ST748, ST451, ST9, ST307 and ST738,
126 the SSI-1 PCR primers failed to amplify. In these strains the 9.7 kb or 1.1 kb PCR amplicons
127 expected for SSI-1 positive and negative strains, respectively, were not observed.
128 Additionally there were 21 serotype 1/2a strains representing ST20, ST121, ST504, ST741,
129 ST749, and ST755 that showed 2.2 kb amplicons consistent with such strains harboring
130 homologs to *L. innocua* genes (*lin0464* and *lin0465*) instead of the five genes comprising the
131 *L. monocytogenes* SSI-1 genes.

132 Resistance to BC was detected in 18% (25/142) of the strains that showed growth at BC
133 concentration of ≥ 10 $\mu\text{g/ml}$. These strains showed MICs of 10 $\mu\text{g/ml}$ (n=3), 15 $\mu\text{g/ml}$ (n=3),
134 20 $\mu\text{g/ml}$ (n=4), 25 $\mu\text{g/ml}$ (n=10) and 30 $\mu\text{g/ml}$ (n=5). PCR analysis showed 20 (80%) and 3
135 (12%) of these strains to harbor *qacH* and *bcrABC*, respectively, whereas these BC resistance

determinants were not detected in 2 (8%) of the BC resistant strains (Table S1). BC resistant strains were distributed between five MLST CC and one singleton. Strains from CC9 (n=2), CC20 (n=1), CC31 (n=1), CC121 (n=15) and ST749 (n=1) harbored *qacH*, whereas *bcrABC* was harbored by strains from CC9 (n=2) and CC31 (n=1). The remaining two BC resistant strains belonged to CC31 (n=1) and CC504 (n=1).

One hundred and twenty nine (91%) strains were classified as poor (CV OD₅₉₅ < 0.2), 11(8%) as medium (CV OD₅₉₅ range 0.2 – 0.35) and 2 (1%) as high (CV OD₅₉₅ > 0.5) biofilm formers. Strains classified as medium and high biofilm formers represented various serotypes and genotypes. Overall there was no correlation detected between biofilm formation capacity and strain serotype, genotype or isolation source.

4. Discussion

Similar to other countries around the world listeriosis remains a significant public health and food safety threat in Switzerland. Food products are the primary source for human infection and an improved understanding of the distribution and characteristics of food associated *L. monocytogenes* is necessary in order to improve our understanding of the potential threat and contribution of various food matrices to human listeriosis transmission. In this study we have characterized 142 *L. monocytogenes* strains that were isolated from different food matrices including meat (63%), milk (24%) and plant (6%) food products and their associated production environments as well as from other (7%) food categories in Switzerland during the time period from 2011 to 2014.

Serotype distribution analysis among these strains revealed that 1/2a was the most prevalent serotype. These observations are in agreement with various previous studies from other countries, which have also found this serotype to predominant among strains isolated from food and food production environments (Gianfranceschi et al., 2009; Parisi et al., 2010;

161 Kramarenk et al., 2013; Martín et al., 2014). In contrast to serotypes 1/2a, 1/2b and 4b that
162 were more evenly distributed among strains recovered from the different food sources
163 examined, the serotype 1/2c strains showed a bias towards meat association. Similar
164 observations have been previously reported by others (Gianfranceschi et al., 2009;
165 Kramarenk et al., 2013; Martín et al., 2014).

166 MLST genotyping grouped the strains into 61 STs that were assigned to 24 clonal complexes
167 and 6 singletons. Genetic lineages assignment showed that lineage II (78% vs 22%) was more
168 prevalent compared to lineage I among the Swiss food associated *L. monocytogenes* strains
169 isolated during this period. These observations are similar to those reported from several other
170 countries showing that lineage II strains are more frequently isolated from food and food
171 associated environments compared to those of lineage I (Autio et al., 2002; Gianfranceschi et
172 al., 2009; Parisi et al. 2010; Kramarenk et al., 2013; Haase et al., 2014; Martín et al., 2014).

173 MLST analysis also showed a high prevalence of CC9 and CC121 in the food associated *L.*
174 *monocytogenes* isolates in Switzerland. These observations are in agreement with those
175 reported by Chenal-Francisque et al. (2011) who described a frequent appearance of these two
176 clonal complexes in many countries. In addition, Parisi et al. (2010) and Martín et al. (2014)
177 have also reported a wide occurrence of ST9 and ST121 in meat-processing environments.

178 PCR based analysis of the 142 strains showed a 50% SSI-1 prevalence among the examined
179 Swiss strains. In agreement with previous reports the SSI-1 in these strains is harboured by
180 non-serogroup 4 strains that mostly belong to genetic lineage II (56.8 %) (Ryan et al., 2010;
181 Hein et al., 2011; Arguedas-Villa et al., 2014). A subset of ST112 serotype 1/2a *L.*
182 *monocytogenes* strains that amplify smaller SSI-1 amplicons (2.2 kb instead of 9.2 kb)
183 because they harbour homologs of *L. innocua* genes *lin0464* and *lin0465* instead of the *L.*
184 *monocytogenes* SSI-1 gene set were previously also reported (Hein et al., 2011; Arguedas-
185 Villa et al., 2014). A similar subset was detected in the current study. But in addition to ST

121 strains this subset also includes serotype 1/2a strains that belong to ST20, ST504, ST741, ST749, and ST755.

Previous studies have documented BC resistance among *L. monocytogenes* strains isolated in various countries (Mullapudi et al., 2008; Ratani et al., 2012; Dutta et al., 2013; Xu et al., 2014). Among Swiss *L. monocytogenes* strains analyzed here a BC resistance prevalence of 18% (25/142) was determined. All BC resistant strains detected belonged to genetic lineage II although other studies have detected BC resistance in both genetic lineage I and II strains (Mullapudi et al., 2008; Ratani et al., 2012; Dutta et al., 2013; Xu et al., 2014). Most of the BC resistance strains detected possessed *qacH* (80%) and *bcrABC* (12%), whereas 8% (2/25) did not harbour either of these two BC resistance determinants. Müller et al. (2013) in their previous study also reported both *qacH* and *bcrABC* harboring BC resistant *L. monocytogenes* strains in their strain collection. Interestingly four CC9 strains resistant to BC were divided into two serogroups. One group harbored *qacH* and belonged to serotype 3c, whilst the other group harbored *bcrABC* and belonged to serotype 1/2c. Meanwhile similar to our observations here BC resistant *L. monocytogenes* strains lacking known resistance determinants such as *qacH* or *bcrABC* have been previously observed (Ortiz et al., 2014). Possible explanations put forward for the increased BC tolerance in such strains include a mutation in endogenous efflux pumps (Romanova et al., 2006; Rakic-Martinez et al., 2011) or modifications in the cell wall that somehow reduce BC access to its cell membrane target (To et al., 2002).

Assessment of biofilm formation capacity revealed that the majority of the strains isolated from the different food matrices are poor biofilm formers. There were, however, a few strains that displayed medium to strong biofilm formation tendency. These findings are similar to those reported by other authors (Harvey et al., 2007; Barbosa et al., 2013). Similar to Harvey et al. (2007) there were no difference in biofilm production capacity detected between strains

derived from different food sources and associated production environments. Furthermore no relationship could be discerned between biofilm formation and strain serotypes or MLST genotypes could be found.

Strains that caused human listeriosis between 2011 and 2013 in Switzerland were recently characterized showing that serotypes 1/2a (62.4 %), 1/2b (5.4 %), 1/2c (2.1 %) and 4b (30.1 %) strains were associated with human infections during this time period (Althaus et al., 2014). In comparison to the current study that characterized *L. monocytogenes* strains associated with different food matrices sampled in an overlapping time period there was a similar high prevalence of serotype 1/2a and 4b strains reflected in food products and the human listeriosis cases. A comparison based on MLST genotypes although showing genetically diverse and overlapping *L. monocytogenes* populations among Swiss clinical cases and food associated strains, there were differences in the predominating genotypes. Although ST9 (n=18) and ST121 (n=14) are the most frequent among food isolates they are less frequent among the human listeriosis isolates. In contrast ST1 and ST8 were the most prevalent sequence types in the human strains collection (Figure 1).

5. Conclusion

The present study delivers insights into the genetic and phenotypic characteristics of food derived *L. monocytogenes* strains occurring in Switzerland. No links between serotypes, lineages or MLST types on the one hand, and the food origin of the strains on the other hand, were found. The MLST sequence types found in Switzerland are largely distributed across the global clonal diversity of *L. monocytogenes*. The results further highlight strain differences in the occurrence of several genetic elements, which are linked to bacterial persistence.

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